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A microscale method of protein extraction from bacteria: Interaction of *Escherichia coli* with cationic microparticles

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ABSTRACT

We developed a simple, highly selective, efficient method for extracting recombinant proteins from *Escherichia coli*. Our recombinant protein yield was equivalent to those obtained with high pressure homogenization, and did not require exposure to harsh thermal, chemical, or other potentially denaturing factors. We first ground conventional resin, designed for the exchange of small anions, into microparticles about 1 μm in size. Then, these cationic microparticles were brought convectively into close contact with bacteria, and cell membranes were rapidly perforated, but solid cell structures were not disrupted. The released soluble components were adsorbed onto the cell wall associated microparticles or diffused directly into the supernatant. Consequently, the selective adsorption and desorption of acidic molecules is built into our extraction method, and replaces the equally effective subsequent capture on anion exchange media. Simultaneously to cell perforation flocculation was induced by the microparticles facilitating separation of cells yet after desorption of proteins with NaCl. Relative to high pressure homogenization, endogenous component release was reduced by up to three orders of magnitude, including DNA, endotoxins, and host cell proteins, particularly outer membrane protein, which indicates the presence of cell debris.

1. Introduction

Extraction of proteins from bacteria without disrupting the cells is a key simplification of conventional purification processes and isolations of labile proteins. Typically, isolation of recombinant proteins from bacteria involves a cascade of operations, including cell harvest, cell disruption, and homogenate clarification to remove cell debris, followed by a combination of chromatographic methods to obtain highly pure protein (Demain and Vaishnav, 2009). Currently, a variety of methods are available for cell disruption, including enzymatic digestion, chemical treatments, and ultrasonication. For preparative scale protein extractions, high pressure homogenization and milling techniques are the preferred methods (Middelberg, 1995). Also, osmotic and thermal shock can be applied as supplementary forces to enhance the performance of other disruption techniques (Harrison, 1991). In all cases, and irrespective of the scale, the removal of generated cell debris is required before further processing the obtained cell homogenate (Balasundaram et al., 2009). Membrane filtration or chromatographic techniques, which commonly follow the disintegration step in a downstream processing routine, are susceptible to blocking and fouling when small particles are present in the process solution. Thus, efficient removal of cell debris or other solid components is difficult. Centrifugation, depth filtration, or a combination thereof is typically applied, but again, difficulties arise with small particle extractions. High product yields can be achieved by increasing the number of disintegration cycles to further fragment the cell debris, but eventually, this leads to an increase in viscosity. Consequently, very high centrifugal forces or large filter units are required, but efficient clarification results in low throughput yields. When working at the laboratory scale, these problems can be solved with high performance equipment; but at preparative, or even more industrial

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scales, this processing step represents a severe bottle-neck. Thus, alternative techniques are required with higher selectivity for extracted components.

Typically, the bacterial cell envelope consists of a plasma membrane and a peptidoglycan cell wall. Gram negative strains, such as *E. coli*, contain a thin peptidoglycan layer and an outer membrane composed of a lipopolysaccharide complex, also known as endotoxin. Endotoxin removal below a critical, nontoxic limit during downstream processing is crucial for any biopharmaceutical product. In general, the integrity and functionality of a cellular system is maintained by the selective molecular sieving properties of its membranes. The bacterial peptidoglycan layer, which provides the shape of the cell, typically allows diffusion of relatively large molecules, due to its loose meshwork (Demchick and Koch, 1996; Vázquez-Laslop et al., 2001); in contrast, cell membranes are generally impermeable to passive transport of macromolecules, like proteins. Chemical or physical methods for increasing cell membrane permeability, e.g., organic solvent and osmotic shock treatments, have been shown to be more selective for the release of intracellular proteins compared to cell disruption methods (Harrison, 2011).

Membranes are composed of amphiphilic molecules that form hydrophobic, introversive domains and hydrophilic chains that are exposed to the aqueous surfaces. The structural stiffness of these so called bilayers is primarily limited, due to the interfacial tension of water, which is excluded from the hydrophobic regions (Hancock et al., 1994; Nikaido, 2003). Such a structure is susceptible to internal and external pressures, depending on the enclosed volume. Moreover, weak interactions between membrane moieties allow lateral movement and transverse rearrangements, which makes the bilayer vulnerable to disaggregation when subjected to surface tension-reducing agents. However, membranes of unicellular specimens which are extensively exposed to environments contain divalent cations, like Ca^{2+} and Mg^{2+}, which increase structural stiffness by minimizing the repulsive forces between the predominantly negatively charged hydrophilic residues (van Loosdrecht et al., 1990). The negative charges on microbial surfaces also promote the electrostatic interaction of these cells with positive charges on anion exchange resin (Terada et al., 2006). The attractive or repulsive force intensities between charges in an aqueous medium depend on the buffer conditions, such as pH and ionic strength, and on the interfacial distance. Previously, it was shown that some electrostatic interactions can perturb membrane integrity and lead to cell inactivation (Gottenbos et al., 2001; Leroueil et al., 2008; Palermo et al., 2011). As a possible perturbation mechanism, it has been proposed that divalent cations, which are crucial for membrane integrity, could be exchanged with a high density of positive charges or neutralized, due to complexation with chelating groups (Poortinga et al., 2002).

Our protein extraction method comprises five steps. First, we prepared cationic microparticles with an average diameter of one micron, by grinding up a strong basic anion exchange resin; second, we homogenously mixed a bacterial cell suspension with the microparticles; third, we incubated the mixture without further agitation; fourth, we resuspended the flocs in an elution buffer; and fifth, we separated the liquid from the solid fraction.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma Aldrich (St. Louis, Missouri, US), Merck (Darmstadt, Germany), or Invitrogen (Carlsbad, CA, US).

2.2. Preparation of microparticles

Ion exchange resins were purchased from Sigma Aldrich. Dowex Marathon A2 is a gel-type, strong basic, anion exchange resin, supported on a polystyrene divinylbenzene polymeric matrix, and functionalized with dimethyl ethanol ammonium. Dowex Marathon MSC is a macroporous, strong acidic, cation exchange resin, functionalized with sulfonic groups. Amberlite IRA458 is a gel-type, strong basic, anion exchange resin supported on a divinylbenzene crosslinked acrylic matrix, and functionalized with trimethyl ammonium. Resins were washed by mixing 20:1 (v/v) with 50% ethanol and 20:1 (v/v) double deionized water (ddH_2O), preconditioned to their sodium or chloride form with 10:1 (v/v) 2 M NaCl for cation exchangers and anion exchangers, respectively. Then, beads were washed 5 times by mixing 20:1 (v/v) with ddH_2O until the supernatant conductivity was below 1 mS/cm and the pH was neutral. Resin beads were then wet-ground with a pestle and mortar, until the main solid fraction had an average diameter of about 1 μm. Grinding of 40 g resin in 120 g ddH_2O was performed with a motor-driven pestle and mortar for 24 h. Particle size was measured by optical microscopy with the Live Cell system (Leica Microsystems, Wetzlar, Germany). Particle size distributions were estimated statistically from an equivalent circular diameter by counting 5000–50,000 discrete optical projections using the image analysis software JMicroVision (Roduit, N., University of Geneva, Switzerland Version 1.2.7). The shapes of ground particles were considered irregular, based on optical projections. Microparticle concentrations were determined by weighing the wet pellet and relating it to the total suspension volume. Suspensions were adjusted by centrifugation on a Heraeus Multifuge X3R with a TX-750 Swinging Bucket Rotor (from Thermo Fisher Scientific, Vienna, Austria) in 50 mL tubes, for 2 h, at 4000 × *g* and 23 °C; wet pellets were resuspended in ddH_2O.

2.3. Cells

E. coli HMS174 cells were batch cultivated in a programmable, logic-controlled reactor with semisynthetic media. The media was supplemented with 2.5 g NH_4Cl and 2.1 g $(NH_4)_2SO_4$ per liter to provide a nitrogen source. Yeast extract was added (0.15 g/g dry cell mass) to accelerate the initial growth. After media sterilization in the bioreactor, the pH was adjusted to 7 with 12.5% ammonia solution. The synthetic media was used for the subsequent fed batch culture. A buffer (3 g KH_2PO_4 and 4.58 g K_2HPO_4 per liter media) was added to provide buffering capacity and to serve as a phosphate and potassium source. The other media components were added in sufficient quantity to produce 1 g of biomass: 0.25 g $C_6H_5Na_3O_7 \times 7H_2O$, 0.10 g $MgSO_4 \times 7H_2O$, 0.02 g $CaCl_2 \times 2H_2O$, 50 μL trace element solution, and 3.3 g $C_6H_{12}O_6 \times 1H_2O$. For cultivations of *E. coli* that expressed recombinant superoxide dismutase (SOD), 4 mg $CuCl_2 \times 2H_2O$ and 3.2 mg $ZnSO_4 \times 7H_2O$ per g dry cell mass were added. The recombinant proteins, green fluorescent protein mutant 3.1 (GFPmut3.1) and SOD, were induced to express with the addition of isopropylthio-β-galactoside (IPTG) at 20 μM/g dry cell mass. Approximately 10 L of cell broth was collected from the fed-batch culture and stored at 4 °C. Extraction and homogenization experiments for GFP and for SOD were started within 1–20 h after the cell broth was collected from the reactor. In that interval, cell suspensions and pellets were stored at 4 °C.

2.4. High pressure homogenization

Cells were collected from fermentation broth by centrifugation (50 mL at 4000 × *g* for 10 min) at 23 °C. Cells were suspended in buffer containing 50 mM TRIS, pH 8.0, and 100 mM NaCl at a concentration of 25% (v/v) cells; then, 100 mL batches of cells were

disrupted with high pressure homogenization by applying two cycles of 100 MPa on a Panda2k homogenizer (GEA Niro-Soavi, Parma, Italy).

2.5. Extraction by microparticles

Protein extraction experiments were performed with aliquots from cell broth or with cells collected and re-suspended in buffer at 23 °C. Actual concentrations of each batch were determined by centrifuging 50 mL suspensions at 4000 × *g* for 10 min at 23 °C, and then weighing the wet cell pellets. For experiments with various cell and salt concentrations, pellets were suspended by vortexing in the respective buffers. Final concentrations of cells, microparticles, buffers, and salt were adjusted by adding 10× buffer stocks to 50% (v/v) suspensions and diluting with ddH_2O to a final working volume of 20 mL. Incubations were performed for up to 3 h at 23 °C in stirred beakers or in static tubes. Both the beakers and tubes had similar height to diameter ratios (about 3:5 cm). For stirred incubations, the mixing intensity was adjusted to 800 rpm with a magnetic stir bar of 0.3 cm × 1 cm. Aliquots of 1 mL were diluted 1:2 with the respective elution buffers for quantifications of released proteins. Then, samples were centrifuged at 8000 × *g* for 1 min in a 5415R centrifuge with an F45-24-11 rotor (from Eppendorf, Hamburg, Germany) in 2 mL tubes, at 23 °C. Finally, the supernatants were collected into clean sample tubes for further investigations.

2.6. Atomic force microscopy

Positively charged microscopic slides (Superfrost Ultra Plus; Thermo Fisher Scientific) were sequentially treated with a 50 mM sucrose solution, deionized water, and a 1% (v/v) diluted suspension of microparticles. Drying was performed after each step at 65 °C for 24 h.

All AFM measurements, except for cell visualization, were performed in dry contact mode with a JPK NanoWizard I atomic force microscope (JPK Instruments AG, Berlin, Germany). The topography of the particles was measured with NP-S10 non-conductive silicon nitride tips (Bruker Corporation, Billerica, Massachusetts, USA), which had a nominal spring constant of 0.12 N/m. Open source software Gwyddion (from David Nečas and Petr Klapetek, Brno, Czech Version 2.3.5) and the data processing software from JPK Instruments were used to analyze surface topography and for polynomial flattening analysis.

2.7. Optical microscopy

Confocal and fluorescence microscopy were performed at the Vienna Institute of Biotechnology on a Leica "Live Cell", wide-field microscope. Samples were diluted to ~1% v/v solids in the respective buffer and visualized at 1000× magnification (Leica HCX PL APO 100× 1.4 oil).

2.8. Cell viability

A L7012 LIVE/DEAD® BacLight™ Bacterial Viability Kit for microscopy and quantitative assays (Invitrogen) was used for viability staining. After incubation, *E. coli* that expressed SOD and cell/resin suspensions were diluted 1:10 in 0.9% (w/w) NaCl. Staining components were premixed at a 1:1 ratio of SYTO 9 dye (3.34 mM) and propidium iodide (20 mM); 3 μL of the staining mixture was added to each 1 mL diluted sample, mixed vigorously, and incubated in the dark at 23 °C for 15 min. After incubation, suspensions were mixed again, and 5 μL was applied to microscope slides. Fluorescence was measured at 1000× magnification through a red and green pass filter (N2.1 and L5; Leica) to detect dead cells with propidium iodide stain and live cells with STYO 9 stain, respectively.

2.9. SDS-PAGE

All samples were heated for 10 min at 100 °C after addition of 25% (v/v) 4× SDS sample buffer (Invitrogen) and 10% (v/v) 2 M DTT. Electrophoresis was performed on 8–12% polyacrylamide gels (Invitrogen) in MES-SDS running buffer at 200 V and a maximum of 400 mA for 60 min. Staining was performed with Coomassie R250 and Bismark BraunR (Sigma Aldrich). Densitometry was performed with optical gel scanning (Epson Perfection Scan V770, 600 dpi, 16 bit grayscale) and evaluated with LumiAnalyst (v3.0 Roche Diagnostics, Basel, Switzerland) software.

2.10. GFP protein standard preparation

The GFPmut3.1 protein standard was prepared from *E. coli* homogenate which was centrifuged for 60 min at 10,000 × *g*, then supernatant was 0.2 μm filtered and purified sequentially on an ÄKTA purifier (from GE Healthcare Life Sciences, Uppsala, Sweden) with anion exchange media (AIEX CaptoQ from GE Healthcare Life Sciences), hydrophobic interaction media (ButylSepharose HP from GE Healthcare Life Sciences), and gel filtration (SuperdexG75 prep. grade from GE Healthcare Life Sciences). Purified protein was denatured in 8 M Urea for 10 min at 100 °C, and concentration was determined from its absorbance at 280 nm. The extinction coefficient was calculated with the Expasy ProtParam Tool (Swiss Institute of Bioinformatics, Lausanne, Switzerland) based on the GFP amino acid sequence (MSKGEELFTGVVXILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLXVXWXTLVTTLXVQCFSRYXDHMKRHDFFKSAMXEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTXIGDGXVLLXDNHYLSTQSALSKDXNEKRDHMVLLEFVTAAGITHGMDELYK). Corresponding fluorescence intensities were determined by standard calibration at 485 nm excitation and 535/20 nm emission on a GENios Pro plate reader (Tecan, Maenedorf, Germany)

2.11. SOD protein standard preparation

The SOD protein standard was prepared by extraction from *E. coli* cells with Marathon A2 microparticles. Cells were incubated for 2 h at ratio 0.7 v/v (resin:cells) in 50 mM TRIS buffer at pH 8.0. Protein was desorbed with 0.2 M NaCl and subsequently purified by gel filtration (SuperdexG75 prep. grade from GE). Purified protein was denatured in 8 M Urea for 10 min at 100 °C, and concentration was determined from the absorbance at 280 nm. The extinction coefficient was calculated with the Expasy ProtParam Tool (from Swiss Institute of Bioinformatics, Lausanne, Switzerland) based on the SOD amino acid sequence (MATKAVCVLKGDGPVQGIINFEQKESNGPVKVWGSIKGLTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHGGPKDEERHVGDLGNVTADKDGVADVSIEDSVISLSGDHCIIGRTLVVHEKADDLGKGGNEESTKTGNAGSRLACGVIGIAQ). Concentrations of samples were evaluated by SDS-PAGE and densitometry to create the 5 point non-linear standard calibration curve.

2.12. Outer membrane protein reference

The heavier solid fraction of *E. coli* homogenate was separated by centrifugation at 4000 × *g* for 15 min. The supernatant was transferred to clean tubes, and the light fraction was collected after 60 min centrifugation at 4000 × *g*. The pellet was suspended in deionized water and washed sequentially two times, as described above. Reference samples of cell debris and crude homogenate were diluted 1:5 with 10 M Urea at pH 8.0 and incubated for 1 h on a rotating shaker.

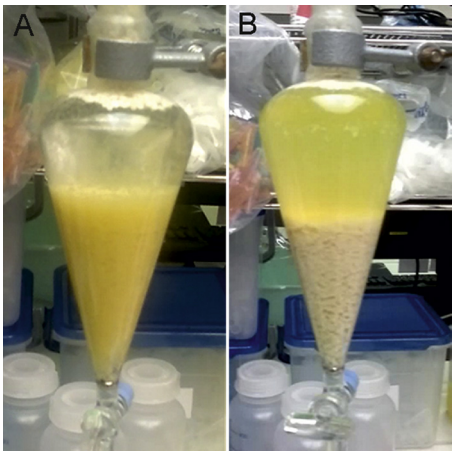

Fig. 1. Extraction of GFP from *E. coli* with Marathon A2 resin microparticles. (A) Resin microparticles were mixed with *E. coli* that expressed green fluorescent protein (GFP); this caused the formation of flocs (yellow), which settled rapidly. (B) After adjusting NaCl to 0.5 M, the GFP protein (green) was released into the solution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.13. DNA and endotoxin determination

DNA was quantified with the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen). Endotoxin was quantified with the PyroGene™ Recombinant Factor C Assay (Lonza, Basel, Switzerland). SOD activity was estimated with the 19160 SOD determination kit (Sigma Aldrich). All supernatant samples were centrifuged at 16,000 × *g* (1 mL) for 30 min at 23 °C, filtered through PVDF membranes (0.2 μm pore size), and diluted at 1:10 with the respective buffer to an effective measurement range. Measurements were performed according to respective kit instruction notes on a GENios Pro or an Infinite 200 M plate reader (Tecan).

3. Results

3.1. Visible effects

Microparticles were prepared by grinding, with a pestle and mortar, a gel-type anion exchange resin (Marathon A2; DOW), which is typically used for industrial demineralization in packed bed systems. This resin comprised spherical beads with an average diameter of 650 μm, composed of cross-linked polystyrene-divinylbenzene, and functionalized with dimethylethanolamine. A charge density of 1.8 mmol/g dry microparticles was determined with acidimetric titration. The size distribution of the microparticles was determined with optical microscopy (Supplemental Fig. 1). We found that the microparticles were irregular in shape. To facilitate protein extraction monitoring, we chose an *E. coli* strain that expressed recombinant green fluorescent protein (GFP) (Reischer et al., 2004) in the cytoplasmic space as a bacterial model system. The extraction process was performed as follows (Supplemental Fig. 2): the *E. coli* cells were collected by centrifugation and resuspended in 50 mM TRIS buffer, pH 8.0. An aqueous suspension of microparticles was then added to the *E. coli*. Floc formation occurred immediately upon mixing microparticles and cells. These flocs settled rapidly, leaving a clarified, uncolored supernatant (Fig. 1A). Flocs were incubated for 1 h at room temperature. Then, the sodium chloride concentration was adjusted to approx. 0.5 M, like a conventional elution buffer, and GFP was released, which colored the supernatant green (Fig. 1B). After release, the pale floc network of microparticles and cells remained stable.

Atomic force microscopy (AFM) analysis was performed to obtain a more detailed view of the structure of the floc network.

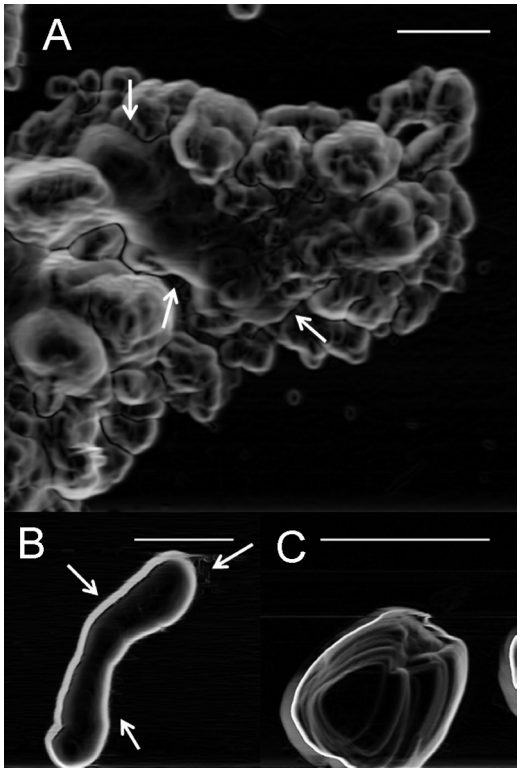

Fig. 2. Comparison of shape and appearance of a fraction of a floc with a single *E. coli* cell and a Marathon A2 microparticle by atomic force microscopy. White arrows indicate the cell boundary. (A) *E. coli* cell entrapped within a layer of microparticles. (B) Single *E. coli* cell suspended in buffer. (C) Single microparticle. Scale bars: 1 μm.

AFM analysis revealed that *E. coli* cells were enclosed by a compact layer of microparticles (Fig. 2A). This observation was also confirmed with optical microscopy (Fig. 3). Actually, the cell shape was not affected by the adhesion of microparticles and network formation, as observed by comparing a single *E. coli* cell (Fig. 2B) and a single microparticle (Fig. 2C). *E. coli* cells expressing GFP could not be assayed for viability, due to background GFP fluorescence, which interfered with the fluorescent dyes used for viability staining. Therefore, we chose another recombinant protein expressed in *E. coli*, human superoxide dismutase (SOD) (Striedner et al., 2003), as a model system. The separated, untreated cells, and those captured within the floc network were stained and analyzed with optical microscopy (Fig. 4). The most striking observation was that the cells were not disintegrated and their initial shapes were unaffected. Surprisingly, many cells inside flocs could be labeled with the viability stain (Fig. 4B).

3.2. Basic conditions during incubation

We studied four main parameters that influenced protein extraction performance during incubation: the incubation time, the ratio of microparticles to cells, and the pH and ionic strengths of the solution. The influence of the incubation time was investigated by periodically sampling the mixture of microparticles and cells; aliquots were analyzed by adjusting the NaCl concentration to 0.5 M NaCl to desorb released protein from the resin, and separating flocs from solution with centrifugation. The microparticle-to-cell ratio was defined as the volumetric ratio of the microparticle pellet to the cell pellet obtained after centrifugation. As a reference, we subjected separate samples to two cycles of high pressure homogenization at 100 MPa to obtain what we considered a 100% yield of soluble protein from *E. coli*.

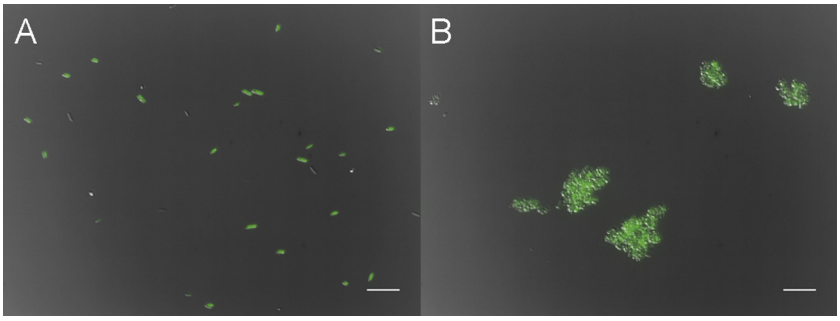

Fig. 3. Fluorescence microscopy of GFP in *E. coli* cells and flocs, formed upon addition of Marathon A2 microparticles. (A) *E. coli* GFP-expressing cells were suspended in 0.05 M TRIS, pH 8.0, and incubated for 1 h. (B) *E. coli* GFP-expressing cells were mixed at 10% (v/v) cells with a 0.7 microparticle-to-cell ratio in 0.05 M TRIS, pH 8.0, and incubated for 1 h. Scale bars: 10 μm.

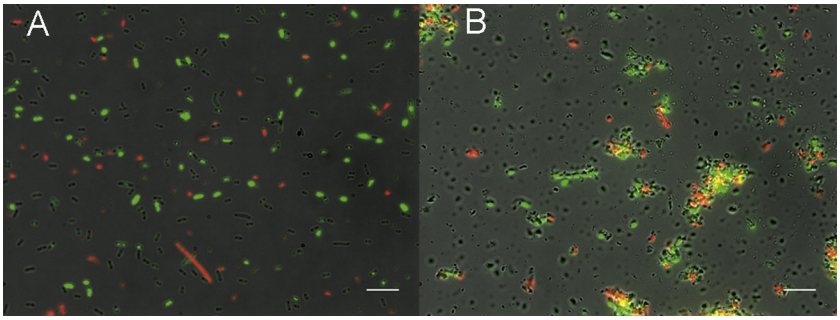

Fig. 4. Fluorescence microscopy of LIVE/DEAD® BacLight™ stained SOD-expressing *E. coli* cells and flocs, which formed upon addition of Marathon A2 microparticles. (A) *E. coli* cells that expressed recombinant SOD were suspended in 0.05 M TRIS, pH 8.0, and incubated for 1 h. LIVE/DEAD® BacLight™ bacterial staining shows live (green) and dead (red) cells. (B) *E. coli* cells that expressed recombinant SOD were suspended at 10% (v/v) cells, mixed with Marathon A2 microparticles at a microparticle-to-cell ratio of 0.7 in 0.05 M TRIS, pH 8.0, and incubated for 1 h. LIVE/DEAD® BacLight™ bacterial staining shows live (green) and dead (red) cells. Scale bars: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

For determining how pH influenced GFP release kinetics and total yield, the *E. coli* fermentation broth was adjusted with NaOH to pH 7.5, 8.0, or 8.5; simultaneously, the physiological ionic strength was maintained at about 12 mS/cm, which corresponded to approximately 0.1 M NaCl. Microparticles were added at ratios of 0.3 (Fig. 5A) or 1.0 (Fig. 5B) to an *E. coli* suspension, with a final concentration of 10% (v/v) cells per wet pellet. The amounts of extracted GFP per volume of wet cell pellet were determined by measuring fluorescence in the supernatant. GFP release kinetics were determined by taking samples between 0.2 and 3.0 h. At pH 8.5, and a microparticle-to-cell ratio of 1.0, the extraction process was complete within 2 h, and yielded an extraction efficiency of 100% with respect to the reference. Overall protein release was more rapid and more efficient at higher pH values. This trend became clearer upon examining the entire experimental data set (Supplemental Fig. 3).

To evaluate how ionic strength influenced protein release during incubation, cells were collected by centrifugation and resuspended in buffers with increasing ionic concentrations between 0.0 (no added NaCl) and 0.5 M NaCl at pH 8.0. At the same time, we examined how the ratio of microparticles to cells affected extraction efficiency by testing ratios between 0.3 and 1.0. Yields were evaluated after 0.2 and 2.0 h of incubation (Fig. 6A and B, respectively). The influence of salt was substantial, but the ratio of microparticles to cells had a minor impact. GFP release kinetics was reduced significantly with increasing salt concentration. Without salt addition, extraction was highly efficient at low ratios and after a short contact time. At low concentrations of salt (0.05 M NaCl), 100% GFP yield was achieved after 2 h. At 0.1 and 0.5 M NaCl, only 60% of the total protein was extracted after 2 h. The osmotic effect of low salt buffer on protein release was only around 10%. The entire data set is shown in Supplemental Fig. 4.

The protein binding capacity of ion exchangers depends on the salt concentration. Therefore, we determined adsorption isotherms by measuring extracted GFP in the supernatant before and after elution, and evaluated the mass balance. This determination was carried out in a series of small scale extraction experiments, where different amounts of microparticles were added to the same amount of cells (Supplemental Fig. 5). At low salt concentrations, adsorption was favorable, and we observed capacities between 20 and 50 mg GFP/mL of microparticles in the pellet and high equilibrium association constants. With increasing salt concentrations, binding strength and capacity dropped dramatically. In fact, at 0.5 M NaCl, the capacity was practically zero. This was a remarkable result, because the yield of released GFP was around 60% after 2 h of incubation.

3.3. Selectivity

The purity of extracted protein samples was analyzed by SDS-PAGE and compared to the protein composition of the high pressure homogenate samples (Fig. 7A). Lanes 3 and 4 represent cell homogenates; the first (lane 3) was treated with 8 M urea to solubilize protein aggregates. Lanes 5–12 show the amounts of protein eluted with increasing salt concentrations; salt was added after incubation for 1 h, and all samples contained a ratio of 0.7 microparticles to cells in 50 mM TRIS buffer, pH 8.0. Recovery of GFP was complete when the elution buffer contained above 0.3 M NaCl. From visual inspection, it is evident that the GFP fractions obtained by extraction with microparticles contained low concentrations of other proteins. In fact, a semi-quantitative analysis with densitometry (Table 1) showed that the amount of *E. coli* host cell proteins was five times lower in extracted samples, in terms of the relative purity of GFP. The cell debris obtained with high pressure homogenization contained outer membrane protein (OMP), which served as a marker protein (lane 2) to indicate the amount of cell

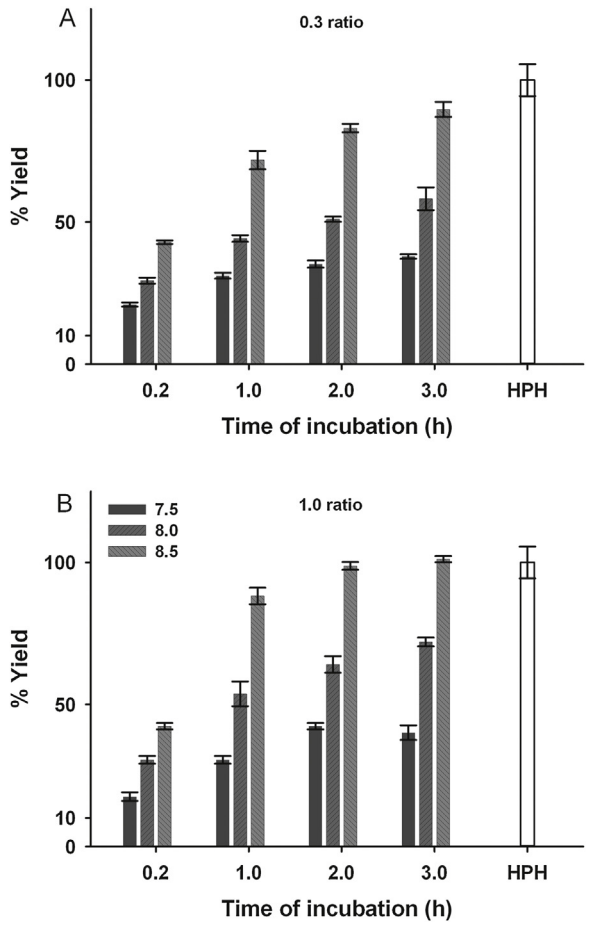

Fig. 5. Influence of starting pH on GFP extraction efficiency and release kinetics. Two different microparticle-to-cell ratios are shown. (A) 0.3 (v/v) and (B) 1.0 (v/v). Increasing gray intensity of bar groups represents increasing pH values of 7.5, 8.0 and 8.5 at the beginning of incubation. High pressure homogenization (HPH) is shown as a reference. Protein extraction yielded close to 100% within 2 h of incubation time. Extraction was slightly more effective and also faster at a microparticle-to-cell ratio of 1.0 (v/v).

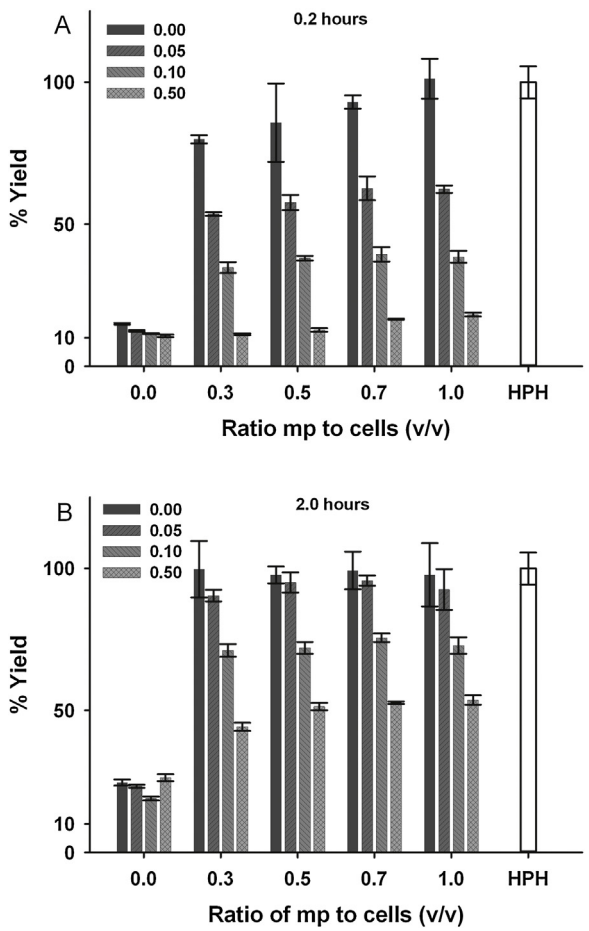

Fig. 6. Influence of starting NaCl concentration on GFP extraction efficiency and release kinetics. Extraction was studied with a microparticle-to-cell ratio between 0 (buffer only) and 1.0 (v/v). Increasing gray intensity of bar groups represents increasing NaCl concentration values of 0.0, 0.05, 0.1 and 0.5 M at the beginning of incubation. Two different incubation times are shown. (A) 0.2 h and (B) 2.0 h. Extraction efficiency decreased with shorter incubation time and higher salt concentration.

fragments in the supernatant. The OMP content in the conventional homogenate was 5 times higher than that observed in samples from the microparticle extraction method.

As a second example, we studied the extraction of recombinant human superoxide dismutase (SOD). In this case, the purity of protein samples obtained by microparticle extraction was compared to those obtained by adsorption of proteins after incubation of microparticles with high pressure homogenate. The protein composition of eluates at 0.5 M NaCl was analyzed by SDS-PAGE (Fig. 7B). Based on densitometry of stained proteins, a significant purification of SOD was achieved by extracting with microparticles, compared to the SOD purified from the untreated cell homogenate and the SOD from the homogenate incubated with the same amount of microparticles as used for cell extraction. Low (<15 kDa) and high (>100 kDa) molecular weight proteins were not detected in microparticle extraction eluates. No significant variation in purity or extraction efficiency was revealed with different ratios of microparticles to cells or with different times of extraction (1–3 h). In addition to the extraction yield and purity on the protein level, we quantitatively determined the amounts of released DNA and endotoxin. After 3 h of incubation, deposited flocs were redispersed, and aliquots were eluted with 0.0 (no added NaCl), 0.5, or 1.0 M NaCl. After elution, flocs were centrifuged and supernatants were analyzed. For comparison, microparticles were added to high

Table 1
Protein distributions after extraction and elution with different NaCl concentrations, determined by fluorescence and densitometry. Yield of protein released by extraction with Marathon A2 microparticles from a 10% (v/v) wet cell pellet suspension after 1 h incubation at a microparticle-to-cell ratio of 0.7 in 50 mM TRIS buffer, pH 8.0. GFP released in the supernatant was quantified by fluorescence (RFU); SDS-PAGE-separated protein bands were quantified by densitometry (BLU). The amounts of released host cell protein (HCP) and outer membrane protein (OMP) were estimated by densitometry (BLU). The reference protein band, with a relative mass of 37 kDa, was detected in the washed cell debris sample, collected after high pressure homogenization of cells (*lane 2*, Fig. 5a). This reference band was identified as an outer membrane porin, protein C (34.4 ± 0.4 kDa) from *E. coli*, with an alignment score of 306 in the Swiss Prot database after trypsin digestion and MALDI TOF mass spectrometry.

	c NaCl (M)	0.2	0.25	0.3	0.35	0.4	0.45	0.5	1.0
Protein distribution (%) from homogenate in lane 3 (Fig. 7A)	GFP (RFU)	72	86	92	96	96	94	94	99
	GFP (BLU)	78	91	96	94	98	98	101	132
	HCP (BLU)	17	17	17	21	21	22	28	31
	OMP (BLU)	8	13	16	17	17	22	18	43

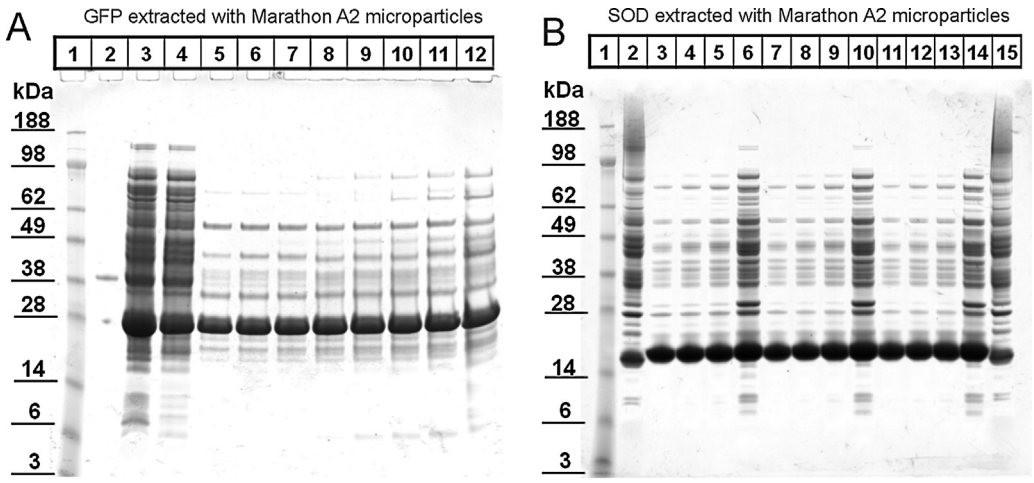

Fig. 7. SDS-PAGE of protein extracted from *E. coli* with Marathon A2 microparticles under different conditions. *E. coli* cells (or equivalent homogenate) were resuspended at 10% (v/v) cells and mixed with a 1.0 microparticle-to-cell ratio in 0.05 M TRIS, pH 8.0, and incubated for 3 h. (A) Protein gel electrophoresis of GFP, obtained by extraction from *E. coli* cells or captured from high pressure homogenate with Marathon A2 microparticles. Extracted proteins are shown for elution with 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50, and 1.0 M NaCl (*lanes 5–12*). Other samples: (*lane 1*) molecular weight standard, (*lane 2*) washed cell debris, solubilized in 8.0 M Urea, (*lane 3*) unfiltered high pressure homogenate, solubilized in 8.0 M Urea, (*lane 4*) filtered high pressure homogenate. (B) Protein gel electrophoresis of SOD extracted from *E. coli* cells or captured from high pressure homogenate with Marathon A2 microparticles. Extracted proteins are shown for mixtures at a microparticle-to-cell ratio of 0.5 after 1, 2, or 3 h (*lanes 3–5, homogenate capture in lane 6*), at a microparticle-to-cell ratio of 0.7 after 1, 2, or 3 h (*lanes 7–9, homogenate capture in lane 10*), and at a microparticle-to-cell ratio of 1.0 after 1, 2, or 3 h (*lanes 11–13, homogenate capture in lane 14*). Extracted amounts of SOD were quantified with a standard calibration method, based on pure SOD. Other samples: (*lane 1*) molecular weight standards; (*lanes 2 and 15*) filtered homogenate.

Table 2
Yield of SOD, DNA, and endotoxin after extraction from *E. coli* cells or after capture from homogenate with Marathon A2 microparticles. *E. coli* cells suspended at 10% cells (v/v) and high pressure homogenate with an equal starting cell concentration were mixed at a 1.0 microparticle-to-cell ratio in 0.05 M TRIS, pH 8.0, and incubated for 3 h. The yields from the reference high pressure homogenization were SOD: 54.8 ± 3.54 mg/g wet cell pellet, DNA: 9.83 ± 1.04 mg/g wet cell pellet, and endotoxin: $182 \times 10^3 \pm 43.5 \times 10^3$ EU/g wet cell pellet. These yields were quantified with standard calibration methods after extraction and desorption in 0.0 (buffer only), 0.5, or 1.0 M NaCl. The analytical error for the SOD yield was calculated within a 95% confidence interval. The analytical errors of the DNA and endotoxin yields were calculated within a 99% confidence interval.

Microparticles to cells ratio (v/v)	Elution buffer	% Yield from homogenate		
		SOD	DNA	Endotoxin
1.0	c NaCl (M)	Average ± error	Average ± error	Average ± error
By extraction from cells	0.0	67.8 ± 19.8	0.17 ± 0.03	0.038 ± 0.011
	0.5	100.7 ± 12.5	0.10 ± 0.02	2.14 ± 0.55
	1.0	100.0 ± 12.5	2.40 ± 0.32	10.9 ± 2.61
By capture from homogenate	0.0	85.4 ± 34.1	0.36 ± 0.05	10.2 ± 4.06
	0.5	100.0 ± 12.7	0.51 ± 0.07	12.7 ± 3.45
	1.0	97.8 ± 12.6	70.8 ± 9.44	61.0 ± 14.7

pressure homogenates under the same experimental conditions; e.g., the same ratio of microparticles (Table 2).

SOD was not completely bound; in fact, two thirds of the released SOD was found in the supernatant, without adding NaCl for elution. At 0.5 and 1.0 M NaCl, 100% of SOD was recovered. From the mass balance analysis, we derived a capacity of 10 mg SOD per mL of microparticles. In contrast, the capture of SOD from the high pressure homogenate was less effective; only 5% of SOD in the homogenate could be bound, corresponding to a capacity of about 2.5 mg/mL of microparticles. With the microparticle extraction method, only 3% of the total DNA and only 10% of the total endotoxin were released, based on desorption with 1 M NaCl. The corresponding values from homogenates were approximately 70% for both compounds. The DNA content in the 0.5 M NaCl eluate from homogenate was fairly low, about 0.5%, but endotoxin levels were 6 times higher in the homogenate eluate than in the microparticle extracted eluate.

4. Discussion

Protein extraction initiated with cationic microparticles is a simple, efficient method that works at physiological or slightly alkaline pH and does not require special equipment. In contrast to mechanical cell disruption methods, microparticle-induced cell perforation does not require energy input. The electrostatic interactions provided by positive charges are sufficient to cause the cell envelope to release cytoplasmic protein. We found that protein extraction was most efficient when microparticles were mixed with cells homogenously and then agitation was stopped, which allowed the floc network to form and settle. Mixing during incubation had a negative effect on the extraction efficiency (Fig. 8A). Also, when whole anion exchange beads or cation exchange (anionic) microparticles were used (Fig. 8B), we did not achieve cell perforation or protein release. Hence, tight adhesion to cells, a high density of positive charges, and accessible surface areas are essential for effective membrane perforation and protein release. Although adhesion did not affect cell shape, the main part of the expressed recombinant protein and a minor portion of host cell protein, DNA, and endotoxin, were adsorbed to the microparticles or were released into the supernatant, respectively.

Based on these observations and previous considerations (Gottenbos et al., 2001; Nikaido, 2003; Terada et al., 2006), the following mechanism of protein extraction is proposed. At physiological pH or above, the cell surface is predominantly negatively charged, which attracts positive charges present on anion exchange surfaces. This interaction favors displacement of

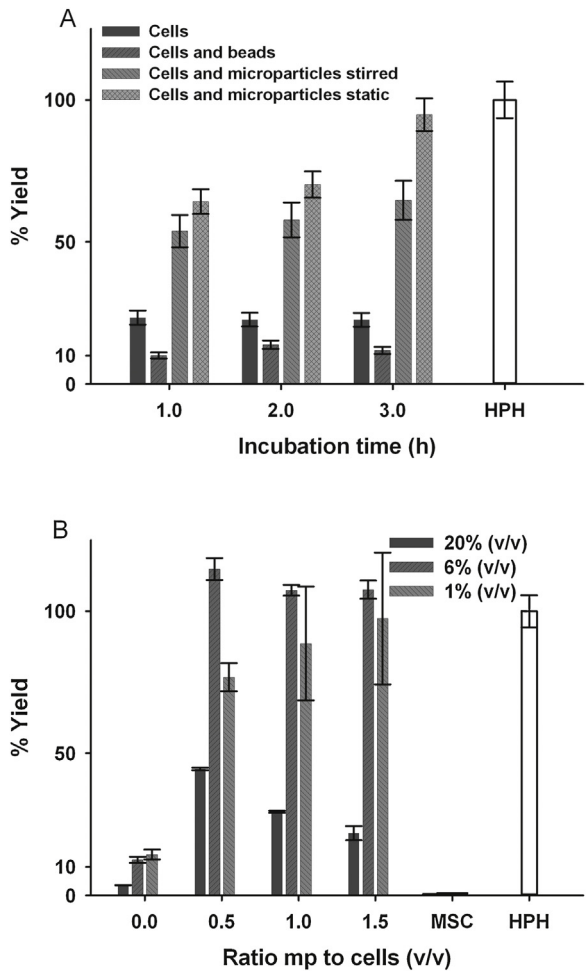

Fig. 8. (A) Yield of SOD after extraction from *E. coli* cells under different mixing and static conditions with Marathon A2. *E. coli* cells, resuspended at 10% (v/v) cells, were mixed with Marathon A2 at ratios of 0.0 (buffer only), 0.7 (unground resin bead-to-cell ratio), or 0.7 (ground microparticle-to-cell ratio) in 50 mM TRIS, pH 8.0. Mixtures were incubated for up to 3.0 h in beakers with stirring. A static incubation setup was performed in parallel with a microparticle-to-cell ratio of 0.7 (v/v). Extracted SOD was eluted with 0.5 M NaCl, and proteins separated by SDS-PAGE were quantified with densitometry. The 100% SOD yield shown in the reference high pressure homogenate (HPH) was 54.8 ± 3.54 mg/g cell pellet. The analytical error of SOD yield was calculated within a 95% confidence interval. (B) Yield of GFP after extraction from *E. coli* cells at various cell concentrations with Marathon A2 microparticles. *E. coli* cells were resuspended at 20%, 6%, and 1% v/v and mixed with microparticles at 0.0 (buffer only), 0.5, 1.0, and 1.5 microparticle-to-cell ratios of Martahon A2 or at a 1.0 microparticle-to-cell ratio of Marathon MSC in 50 mM TRIS, pH 8.0, buffer. Mixtures were incubated for 1.0 h. After elution with 0.5 M NaCl, extracted GFP was quantified by fluorescence. The 100% GFP yield shown in the reference high pressure homogenate (HPH) was 25.5 ± 1.43 mg/g cell pellet. The analytical error of GFP yield was calculated within a 99% confidence interval.

membrane-stabilizing cations; when microparticles bind to exposed phosphate and carboxyl groups (Murata et al., 2007), membrane perforation occurs, due to a distortion of the amphiphilic bilayer. Thus, permeability of the whole cell envelope is increased, and proteins are permitted to leak out. However, the structural integrity of the peptidoglycan layer is not affected; the shapes of the cells are maintained, and its molecular sieving properties remain functional; this prevents very large molecules, like DNA, to diffuse readily out of the cell. Thus, the developed method can be regarded as a special type of ultrafiltration, driven by an electrochemical gradient. This method takes advantage of the large surface area of the peptidoglycan layer, which provides the ultrafiltration barrier, and also the very short diffusion paths from the cytoplasmic space to the exterior; thus, each cell represents a distinct microscopic ultrafiltration unit. Consequently, the existing cell structures are used biochemically, to produce the desired protein, and also biophysically, for its recovery and purification.

Overall, extraction of the recombinant protein expressed in the cytoplasmic space was highly effective; we achieved up to 100% of the yield achieved with high pressure homogenization. At the same time, the surprisingly large number of intact cells may not necessarily mean that these cells remained active, but it directly showed that the extraction method was not invasive with respect to structural cell integrity. This result is in good agreement with the determined purity of extracted proteins. The extracted protein showed low amounts of host cell protein, DNA, and endotoxin, and the supernatant had low amounts of cell debris. Experimental results derived from variations in conditions during incubation also supported the proposed mechanism. Stirring led to lower extraction efficiency; this can be explained by the fact that, when the floc network is dispersed, the average distance between cells and microparticles is increased, thereby lowering the effective contact time. Extraction was optimal at high pH and low salt concentration, consistent with the nature of the participating functional groups. Remarkably, extraction was also efficient at higher salt concentration; this finding does not contradict the proposed mechanism, because it was previously shown that the surface charge of *E. coli* retained a negative potential (van Loosdrecht et al., 1990) under high salt conditions. The reaction time of the entire extraction process varied from several minutes to a few hours, consistent with the time scale expected for electrochemical and diffusive processes.

The total volume occupied by solids, cells, and microparticles represents a physical limit for the applicability of the method, because the viscosity of a solution increases according to a power law, particularly when adhesive forces between particles are involved. Thus, efficient mixing of cells and microparticles before incubation, which is required to create a homogeneous floc network, becomes gradually more difficult with increasing cell densities. At a volumetric cell concentration of 20%, the extraction efficiency after 1 h was about 50% with a microparticle-to-cell ratio of 0.5 (Fig. 8B). The respective experimental series with increasing cell densities indicated that efficiency increased with lower microparticle-to-cell ratios. It must be considered that, under the investigated conditions, the ratio did not significantly influence the total yield, and that we never reached a lower limit of the effective microparticle-to-cell ratio in our experiments. Furthermore, for these particular experiments, mixing was performed with a magnetic stirrer or a vortexer, which are unsuitable methods for providing homogeneity of viscous suspensions. Theoretically, smaller microparticles, with a higher surface per volume would make it possible to reduce the volumetric ratio, and concomitantly, the viscosity of the mixture.

Apart from the intrinsic perforation and protein release process, we observed adsorption of acidic intracellular components to the microparticles. This process was characterized by the physicochemical properties of the involved biomolecules and the anion exchange microparticles. Adsorption was determined by the isoelectric points, binding affinity, and equilibrium capacity of the macromolecules involved. The two investigated proteins have low isoelectric points, which favored binding to anion exchangers. DNA and endotoxins, both highly negatively charged, showed strong binding, and could be separated from the protein in a step desorption process with defined salt concentrations. As known from conventional ion exchange chromatography, proteins may have significantly different binding capacities, despite similar sizes and theoretical isoelectric points (Hahn, 2012). This phenomenon was observed for GFP and SOD. For the given expression titer of GFP and the amount of microparticles added, complete adsorption was achieved. For SOD, only low amounts of protein could be adsorbed, despite the higher expression titer compared to GFP. Therefore,

during the extraction process, maximum binding capacity was far exceeded; however, 100% of SOD was released and recovered, whereas the main protein fraction was recovered in the supernatant without adding salt. Experimental series with another type of cationic microparticle, based on an acrylic polymer matrix, had higher binding capacities for SOD (about 50 mg/mL resin), but was not as effective as styrenic microparticles; those microparticles achieved 70% of the maximum extraction yield (Supplemental Figs. 6 and 7). Presumably, if a basic recombinant protein were overexpressed, the entire fraction would be directly released into the supernatant, because it was shown that the cell perforation process is independent of the protein adsorption capacity of microparticles.

Comprehensive reviews on product release strategies (Balasundaram et al., 2009; Grabski, 2009; Harrison, 2011; Peternel, 2013), have indicated that innovations are needed to create integrated technologies and improve selective product release. In particular, there is also a need to alleviate the burden of subsequent unit operations and to de-bottleneck downstream processing. In our opinion, we have provided a method that can fulfill these requirements: protein extraction with microparticles is highly selective and does not create cell debris, which is difficult to remove. Compared to other methods, quantitative yields can be achieved economically, due to the low energy input and short processing times required. Additionally cationic microparticles adhered to cells and immobilized them in flocs, due to immediate electrostatic interactions. Thus, the intrinsic microscale process of protein release proceeded simultaneously and distinctively in each floc cluster, which allowed an iterative upscaling of the setup (Titchener-Hooker et al., 2008). Protein extraction with microparticle technology, followed by separation of the generated flocs, and clarification of the supernatant by sedimentation or filtration is potentially much more effective than conventional downstream processing, which comprises cell disruption, homogenate clarification, and protein capture. Moreover, in laboratory practice, the method is easily applicable for both analytical and preparative purposes. Regarding the upscale of the preparation of the microparticles, initial studies have shown that a combination of a pin mill and ball mill grinding process is capable to produce microparticles in the desired particle size range in a 100 kg scale (data not shown). The resin costs in bulk amounts is very low at around 10–50$ per kg depending on the resin type. With respect to process intensification continuous protein extraction is possible at laboratory and industrial scales.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2015.04.023

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